Applicants: Philip O. Livingston and Friedhelm Helling

Serial No.: 08/475,784 Filed: June 7, 1995

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Please amend the subject application as follows:

## Amendments to the Specification:

Please replace the paragraph beginning at page 65, line 30, with the following amended paragraph:

Immunological adjuvants: DETOX was produced and supplied by Ribi Immunochem Research Inc. (Hamilton, formulated as a lyophilized oil droplet emulsion. It consists of cell wall skeletons (CWS) from bacille Calmette-Guorin and monophosphoryl lipid A (MPLA) from Salmonella minnesota R595. the day of vaccination, 0.25 ml DETOX (250µg CWS and  $25\mu g$  MPLA) was mixed with the GM2-KLH preparation. The vaccine (final volume 0.75ml) was vortexed for 2-3 minutes and administered to the patients within 15 min. BCG was purchased from Bionetics Research Inc. (Rockville, MD). On the day of vaccination, 107 viable units of BCG in 0.1 ml normal saline were added to the GM2-KLH vaccine in each individual syringe (final volume 0.6 ml). The contents were mixed and administered to the patients within 15 min. QS-21 adjuvant (a homogeneous saponin purified from the bark of Quillaja saponaria Molina (10, 11) kindly provided by Cambridge Biotech Inc. (Worcester, MA). To obtain QS-21, the procedure of Kensil et al, 1991 (10) may be employed. Specifically, coarsely chopped Q. saponaria bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h.

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centrifuged and the supernatant extract was containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and two supernatants were pooled. the To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w:v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mΜ acetic acid with the hemolytic activity retained by the dialysis membrane. The hemolytic fraction was lyophilized and redissolved at a concentration of 200 .mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E.M. Science, Gibbston, NJ; 40 to 63 µM particle size, 2.5 cm I.D. x 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. elution of saponins was monitored by carbohydrate assay. Fractions containing the saponins interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in methanol and loaded on a semipreparative HPLC column (Vydac  $C_4$ , 5 $\mu$ m particle size, 3000 nm pore size, 10 mm I.D. X 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and

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individually rotavapped to dryness. Purity of saponins was assessed by analytic HPLC (Vydac C4, 5µm particle size, 3000 nm pore size, 4.6 mm I.D. X 25 cm length) with a gradient of 0.1% TFA in acetonitrile. As described in Newman et al., 1992 (11), QS-21 is defined as the adjuvant active reverse phase HPLC fraction 21 from Q. Saponaria bark extract. 100µg or 200 µg of QS-21 were diluted in 0.25ml normal saline and mixed with GM2-KLH. The vaccine (final volume 0.75ml) was vortexed for 2-3 minutes and administered within 15 min.